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ORIGINAL ARTICLE

Fe(III) stimulates 3-methylindole and 4-methylphenol production in swine lagoon enrichments and *Clostridium scatologenes* ATCC 25775

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Abstract

Aims: To determine the effects of anaerobic electron acceptors on 3-methylindole (3-MI) and 4-methylphenol (4-MP) production in swine lagoon enrichments and *Clostridium scatologenes* ATCC 25775.

Methods and Results: Swine lagoon sediment was incubated anaerobically in tryptone-yeast extract medium with (10 mmol l^{-1}) Na_2SO_4, KNO_3, dimethyl sulfoxide or Fe(III). With Fe(III), 3-MI and 4-MP levels increased significantly to 138 ± 15·8 and 187 ± 14·0 μmol l^{-1}, respectively. *Clostridium scatologenes* cultured in brain–heart infusion medium amended with (10 mmol l^{-1}) Na_2SO_4, KNO_3, MnO_2 or Fe(III), resulted in only Fe(III) significantly increasing 3-MI (1308 μmol l^{-1}) and 4-MP (367 μmol l^{-1}) levels. In semi-defined medium, Fe(III) alone and Fe(III) + L-tryptophan (1 mmol l^{-1}) resulted in a 1·85-fold and 15·6-fold increase in 3-MI levels over L-tryptophan alone, respectively. Fe(III) alone and Fe(III) + L-tyrosine (1 mmol l^{-1}) caused a 4·4-fold and 22·9-fold increase in 4-MP levels over tyrosine alone, respectively. Fe(III) did not increase growth of *Cl. scatologenes*.

Conclusions: Fe(III) increases 3-MI and 4-MP in swine lagoon enrichments and *Cl. scatologenes* broth cultures.

Significance and Impact of the Study: Previous studies suggest Fe(III) addition to swine lagoons could remediate malodorous volatile fatty acids; however, here data suggest Fe(III) could increase malodorous indolic and phenolic levels.

Introduction

Malodorous emissions from animal production and waste facilities are an increasing problem facing the food animal industry. In the swine industry, two important malodorants are 3-methylindole (skatole; 3-MI) and 4-methylphenol (p-cresol; 4-MP) (Mackie *et al.* 1998), which are produced by similar degradation pathways of tryptophan and tyrosine, respectively (Smith and Macfarlane 1997; Whitehead *et al.* 2008). For example, tryptophan is first deaminated to indole pyruvic acid, decarboxylated to yield indole acetic acid and then decarboxylated to 3-MI. The enzymes of either pathway remain largely unstudied except for the final step in 4-MP production, which is known to be catalysed by a glycyl radical enzyme (Selmer and Andrei 2001).

The second step of either the 3-MI or 4-MP pathway is an oxidation of the amino acid alpha-carbon, which suggests the cell may require disposal of the resulting reducing equivalents to facilitate production of the malodorant. This possibility prompted us to test the effect of anaerobic electron acceptors on production of 3-MI and 4-MP, in both blended swine lagoon enrichments and *Clostridium scatologenes* ATCC 25775. *Clostridium scatologenes* ATCC 25775 produces both 3-MI and 4-MP, thus is a useful model organism to study both processes.

Materials and methods

Media, cultures and growth and analytical measurements

Media contained (l^{-1}): 1·0 mg resazurin, 2·0 mg hemin and 1·0 g cysteine-HCl (Holdeman *et al.* 1977). Additional ingredients (l^{-1}) in TY medium were: 20 g tryptone (Fisher Scientific Inc., Waltham, MA, USA), 10 g yeast extract, 40 ml salt solution (Holdeman *et al.* 1977), 1 μl Vitamin K_1 (Sigma Chemical Co. St Louis, MO, USA). Additional ingredients (l^{-1}) in brain–heart infusion (BHI) were: 37 g BHI including glucose (Bacto Becton Dickinson, Franklin Lakes, NJ, USA), 2 g fructose, 10 g yeast extract (Bacto), 2·69 g sodium bicarbonate and 40 ml salt solution (Holdeman *et al.* 1977). Additional ingredients (l^{-1}) in semi-defined casamino acids (SD-CAA), medium were 10 g casamino acids (Difco), 2 g yeast extract, 5 g ammonium sulfate, 2·69 g sodium bicarbonate, 5 ml each of mineral solution no.1 and no. 2 (Bryant and Burkey 1953) and 1 ml modified trace mineral solution (McInerney *et al.* 1979; Genthner *et al.* 1981).

Stock aqueous solutions of indole acetic acid (IAA), 4-hydroxyphenylacetic acid (4-HPAA), Na_2SO_4, KNO_3, dimethyl sulfoxide (DMSO), MnO_2, $FeCl_3$, or water alone were autoclaved then placed overnight in an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lakes, MI, USA) with a 95% carbon dioxide and 5% hydrogen atmosphere. To prepare Fe(III), aqueous $FeCl_3$ were adjusted to 7·0 using NaOH prior to autoclaving. Aqueous L-tryptophan and L-tyrosine was filter sterilized and then flushed with nitrogen gas or placed in the anaerobic chamber to remove oxygen.

Clostridium scatologenes ATCC 25775 was obtained from American Type Culture Collection (Manassas, VA, USA) and passed no fewer than three times in the respective media before experiments were initiated. Cell growth was enumerated using direct microscopic examination in a Petroff-Hausser chamber. HCl-extractable Fe(II) was determined spectrophotometrically using the ferrozine method as described previously (Lovley and Phillips 1986). Determination of pH values was achieved using a Beckman model Φ350 pH meter (Beckman Instruments, Fullerton, CA, USA).

Swine lagoon enrichments

Lagoon sample was retrieved from the Western Kentucky University's swine facility by completely filling a 1-l plastic bottle with lagoon sediment. The sample was immediately transported to the laboratory and homogenized in a Waring blender (Waring Products, Torrington, CT, USA) for 5 min at low speed. One millilitre of the slurry was added to 100 ml of amended TY medium followed by incubation at room temperature. Amended TY medium contained (10 mmol l^{-1} final concentration) IAA, 4-HPAA, Na_2SO_4, KNO_3, DMSO, MnO_2 or Fe(III). Control cultures received 1 ml of sterile anaerobic water. Each condition was performed in triplicate. Samples were removed at 3 days, 1, 2, 3, 4 and 5 weeks and frozen in 1 l aliquots at −20°C until analysis. Malodorant concentrations were determined using the GC/MS with poly(dimethylsiloxane)-coated stir bar sorptive extraction as described previously (Loughrin 2006).

Clostridium scatologenes ATCC 25775 experiments

Clostridium scatologenes ATCC 25775 was cultured in triplicate in BHI medium amended with (10 mmol l^{-1} final concentration) Na_2SO_4, KNO_3, Fe(III) and MnO_2 stock solutions at 37°C. For the experiments conducted using BHI, samples were analysed as described previously (Doerner *et al.* 2009). For experiments conducted in SD-CAA medium, tryptophan or tyrosine was included at 1 mmol l^{-1} and Fe(III) was included 10 mmol l^{-1} final concentration. 3-MI and 4-MP were measured in whole culture using high pressure liquid chromatography as described previously (K.C. Doerner *et al.*, unpublished data) with the following modifications. Briefly, one volume of acetonitrile : methanol (80 : 20) was added to the sample and incubated at ambient temperature for 30 min with occasional vortexing then centrifuged (16 000 ***g***; 10 min; 4°C). Samples were chilled at −20°C for 20 min, centrifuged, then filtered through a 0·45-μm pore size membrane (Fisher Scientific Inc). Samples were analysed immediately or stored at −20°C. A Sunfire C-18 reverse phase column (4·6 × 150 mm; Waters Corp.) was equilibrated in 0·05 mol l^{-1} ammonium acetate (pH 6·0) : acetonitrile (90 : 10) and eluted using a 10-min linear gradient to 100% acetonitrile. 3-MI was detected using excitation and emission wavelengths of 275 and 348 nm, respectively. 4-MP was detected using excitation and emission wavelengths of 276 and 303 nm, respectively.

Statistics

Data are reported mean ± SE. Unpaired *t*-tests were performed using the Microsoft Office 2003 software package. Statistical significance is $P \leq 0{\cdot}05$.

Results

Swine lagoon enrichments

3-MI, 4-MP and indole levels resulting from swine lagoon cultured in TY medium amended with 10 mmol l^{-1} each

of IAA, 4-HPAA or Fe(III) are shown in Fig. 1. Figure 1a shows 3-MI levels increased significantly in the presence of Fe(III) to 138 ± 15·8 μmol l^{-1} 3-MI by day 28 and decreased to control levels by day 35. Addition of IAA did not alter 3-MI levels. Figure 1b shows 4-MP levels were highest when swine lagoon sediment was cultured in the presence of 4-HPAA. Levels rose to 914 ± 123 μmol l^{-1} 4-MP by day 3 and eventually rising to 2927 ± 222 μmol l^{-1}. For Fe(III) addition, 4-MP levels on day 35 were 187 ± 14 μmol l^{-1} and significantly greater than control levels (123 ± 32 μmol l^{-1}).

Of all conditions tested, indole levels increased the greatest in response to IAA amendment (Fig. 1c). Indole levels reached 1023 ± 34 μmol l^{-1} at day 21 and remained high until termination of the experiment. The addition of 10 mmol l^{-1} each of the other alternative electron acceptors DMSO, sodium sulfate or potassium nitrate did not significantly affect levels of any malodorant tested. Levels of phenol and 4-ethylphenol in cultured swine lagoon slurry were not different from controls in any amended medium (data not shown).

Maximum Fe(II) levels were found at day 3 and remain unchanged until day 35. Day 35 values for Fe(II) (mmol l^{-1}) were: control, 1·07 ± 0·18; IAA, 1·16 ± 0·06; 4-HPAA, 1·16 ± 0·21; Na_2SO_4, 0·88 ± 0·11; KNO_3, 1·38 ± 0·45; DMSO, 1·35 ± 0·23 and Fe(III), 8·58 ± 0·28.

Clostridium scatologenes ATCC 25775

The effect of 10 mmol l^{-1} each of sodium sulfate, potassium nitrate, Fe(III) or manganese oxide on the production of 3-MI and 4-MP was tested in *Cl. scatologenes* ATCC 25775 cultured in BHI medium (Fig. 2). 3-MI production was maximum when cultured with Fe(III). At 48 h of incubation, 3-MI was 1308 ± 24 μmol l^{-1} and significantly different than control (911 ± 36 μmol l^{-1}). Fe(III) amendment also resulted in significantly higher 4-MP levels. At 72 h of incubation, Fe(III) amendment and control levels were 367 ± 27 and 252 ± 21 μmol l^{-1}, respectively. Other electron acceptors did not have a similar effect. Sodium sulfate, potassium nitrate and manganese oxide amendment did not result in elevated 3-MI and 4-MP levels compared with control cultures.

The effects of Fe(III) amendment on growth, Fe(II), 3-MI and 4-MP were determined in semi-defined medium containing casamino acids (Fig. 3). Cells grew well in all media tested and were in stationary phase by day 3. Cell density values for day 3 were (10^7 cells per millilitre): SD-CAA (control), 92·5 ± 8·4; tryptophan, 99·0 ± 7·9; tryptophan plus Fe(III), 103·5 ± 1·7; Fe(III), 120·5 ± 6·3; tyrosine, 124·0 ± 9·8 and tyrosine plus Fe(III), 114·5 ± 15·5. In all conditions containing Fe(III), *Cl. scatologenes* ATCC 25775 reduced Fe(III) to Fe(II) to a final concentration of near 10 mmol l^{-1} by 3 days (Fig. 3a). Fe(II) concentration was approx. 2 mmol l^{-1} at day 0 because of the carryover from the initial inocula. Control studies of sterile anaerobic TY medium with

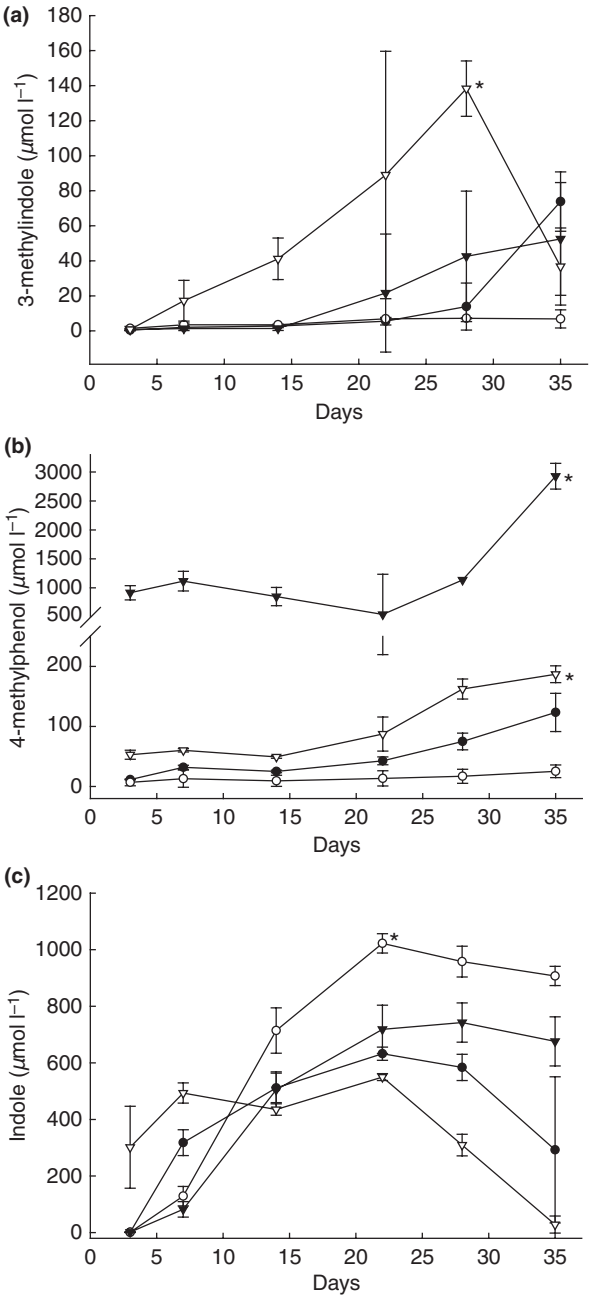

Figure 1 Levels of 3-methylindole (a), 4-methylphenol (b) and indole (c) produced by blended swine lagoon enrichments cultured using tryptone-yeast extract broth at ambient temperature. Amendments (10 mmol l^{-1} each) include: control (●); indoleacetic acid (○); 4-hydroxyphenylacetic acid (▼) and Fe(III) (▽). Values are mean ± SEM. *indicates significantly different from control ($P \leq 0{\cdot}05$) at the time point indicated.

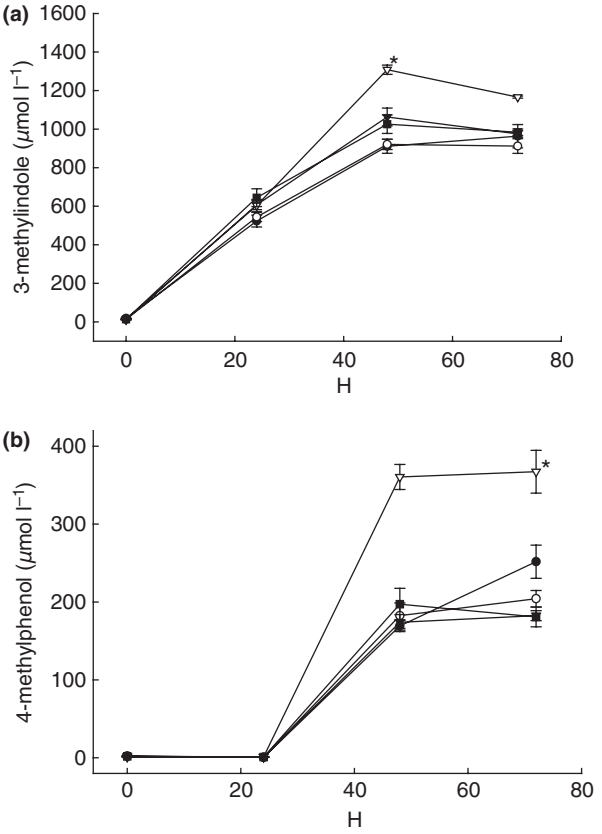

Figure 2 Levels of 3-methylindole (a) and 4-methylphenol (b) produced by *Cl. scatologenes* ATCC 25775 cultured at 37°C in brain–heart infusion broth. Amendments include (10 mmol l^{-1} each): control (●); Na_2SO_4 (○); KNO_3 (▼); Fe(III) (▽) and MnO_2 (■). Values are mean ± SEM. *indicates significantly different from control ($P \leq 0{\cdot}05$) at the time point indicated.

10 mmol l^{-1} Fe(III) incubated for no less than 3 days at 37°C resulted in Fe(II) levels of 0·95 ± 0·1 mmol l^{-1}.

Both 3-MI and 4-MP values rose significantly in response to Fe(III) (Fig. 3). By day 3, 3-MI levels standardized against cell growth, indicated that Fe(III) alone generated 1·85-fold greater 3-MI levels than tryptophan alone, whereas the combination of Fe(III) and tryptophan produced a 15·6-fold increase. Similarly, 4-MP levels increased in cultures incubated with Fe(III) (Fig. 3c). Final concentrations of 4-MP increased by 4·4-fold when only Fe(III) was present in the medium compared with tyrosine alone and a 22·9-fold increase when tyrosine and Fe(III) were combined in the medium.

Discussion

Data presented here indicate inclusion of Fe(III) in blended swine lagoon sediment enrichments increased 3-MI and 4-MP production. The other potential electron acceptors DMSO, sodium sulfate or potassium nitrate did not alter levels of these malodorants. For control purposes, we included 4-HPAA and IAA as amendment in the swine lagoon enrichments. 4-HPAA and IAA are the penultimate compounds in 4-MP and 3-MI production; thus, elevated levels of the respective malodorants were expected by inclusion of these compounds. As expected, 4-HPAA amendment resulted in increased levels of 4-MP produced (Fig. 1b). Surprisingly, however, addition of IAA did not result in elevated levels of 3-MI (Fig. 1a), but instead resulted in elevated indole levels (Fig. 1c). The reason for this is unclear. Nevertheless, Fe(III) addition significantly increased 3-MI and 4-MP levels in blended swine lagoon sediment enrichments.

The mechanism by which Fe(III) increases 3-MI and 4-MP in swine waste lagoon sediments is of interest. Volatile fatty acid levels decrease upon inclusion of Fe(III) in swine waste lagoon sediment because of the family Geobacteraceae present at up to 10^5 cells per gram (Coates *et al.* 2005). Strain NU, a member of this family, was isolated from a swine waste lagoon and displayed dissimilatory Fe(III) reduction at the expense of volatile fatty acids (Coates *et al.* 2005). These experiments indicate that oxidative processes in swine waste lagoons can be facilitated by inclusion of Fe(III). In experiments presented here, Fe(III) was reduced to Fe(II) within 3 days, although levels of 3-MI and 4-MP did not peak until days 28 and 35, respectively, suggesting Fe(III) reduction was not directly coupled to 3-MI and 4-MP production. Nevertheless, it remains possible dissimilatory Fe(III) reducing bacteria are producing 3-MI and 4-MP in swine waste lagoons.

Alternatively, the presence of extracellular substances could potentially act as intermediary electron acceptors between the cell and Fe(III). Humic substances are macromolecular, extracellular matter derived from plant and microbial materials, which contain quinone moieties (Scott *et al.* 1998). This material acts as an oxidant in anaerobic systems accepting electrons from microorganisms which, in turn, abiologically reduce Fe(III) to Fe(II) (Lovley *et al.* 1996). If, however, such a process was taking place in these experiments, levels of other bacterial end products would also be expected to increase. This was not observed as levels of phenol and 4-ethylphenol were unaffected by inclusion of Fe(III).

Fe(III) addition also increased 3-MI and 4-MP production by *Cl. scatologenes* ATCC 25775. When cultured in BHI medium, both malodorants were significantly increased upon Fe(III) inclusion, whereas addition of sodium sulfate and potassium nitrate, and manganese oxide did not affect malodorant levels (Fig. 2). BHI is a rich medium containing many ingredients of unknown concentrations, which complicates physiology studies. For this reason, use of an austere medium having fewer

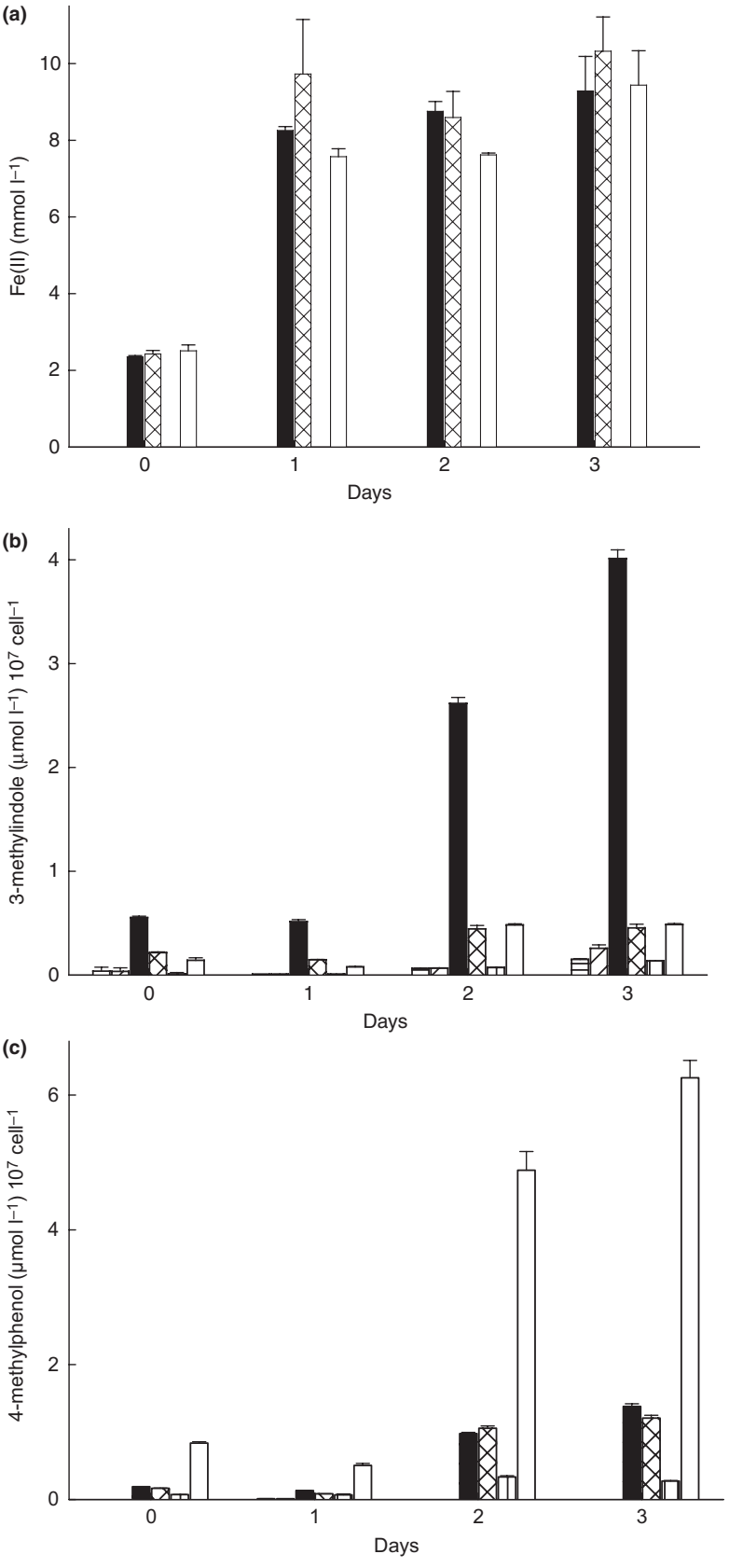

Figure 3 Levels of Fe(II) (a), 3-methylindole (b) and 4-methylphenol (c) produced by *Cl. scatologenes* ATCC 25 775 cultured at 37°C in semi-defined medium with casamino acids. Amendments include: Control, horizontal hatch; L-tryptophan (1 mmol l^{-1}), angled hatch; L-tryptophan and Fe(III) (10 mmol l^{-1}), solid; Fe(III), cross hatch; L-tyrosine (1 mmol l^{-1}), vertical hatch; L-tyrosine and Fe(III), open. Values are mean ± SEM.

ingredients is desirable. A defined medium for *Cl. scatologenes* ATCC 25775 is not available so a semi-defined medium containing yeast extract and casamino acids was used to further test the effects of Fe(III). In SD-CAA, Fe(III) substantially increased 3-MI and 4-MP levels. The addition of Fe(III) was more effective at increasing 3-MI and 4-MP levels than addition of tryptophan or tyrosine alone, the precursors to the malodorants. When each of the respective amino acids was included with Fe(III), a synergistic increase in malodorant level was observed.

The mechanism is unclear by which *Cl. scatologenes* ATCC 25775 is oxidizing tryptophan and tyrosine to 3-MI and 4-MP and reducing Fe(III) to Fe(II). Fe(III) reduction could be achieved via direct or indirect interactions between the cell and iron. A direct interaction, dissimilatory Fe(III) reduction, implies that cell growth would be increased because of the use of Fe(III) as an electron acceptor. Reduction of Fe(III) would allow the cell to oxidize reduced electron carriers, allowing for more efficient oxidation of energy sources, resulting in greater cell density. Fe(III) reduction is rare in fermenting anaerobic micro-organisms and when present constitutes only a minor metabolic pathway (Lovley 1991). However, a member of the order Clostridiales, has been reported to obligately reduce Fe(III) for the oxidation of L-valine or *n*-propanol (Slobodkin *et al.* 2006), indicating dissimilatory Fe(III) reduction is present in clostridia. Here, the addition of Fe(III) did not increase *Cl. scatologenes* ATCC 25775 cell density, suggesting Fe(III) reduction did not benefit cell growth. It is possible, however, *Cl. scatologenes* ATCC 25775 is a Fe(III)-reducing bacterium and Fe(III) was added at insufficient levels and/or the methods employed here were insufficient to detect modest increases in cell density. Alternatively, Fe(III) could be reduced by the anaerobic medium, itself, although control experiments of incubating sterile TY medium with 10 mmol l^{-1} Fe(III) resulted in only 10% reduction to Fe(II).

In conclusion, swine lagoon enrichments and *Cl. scatologenes* ATCC 25775 each produce significantly more 3-MI and 4-MP when cultured in the presence of Fe(III). The mechanism by which Fe(III) reduction results in elevated levels of 3-MI and 4-MP is unclear. Fe(III)-reducing bacteria could be responsible for production of these malodorants in swine lagoons, thus isolation of the organisms should be possible. It is an ongoing effort of this laboratory to isolate such organisms. In addition, it remains possible that *Cl. scatologenes* ATCC 25775 is a Fe(III)-reducing bacterium and iron reduction facilitates 3-MI and 4-MP production. Previous studies suggest Fe(III) addition to swine waste could mitigate malodors because of volatile fatty acids (Coates *et al.* 2005); however, this study suggests Fe(III) addition could increase malodors because of increased 3-MI and 4-MP production.

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